AWARD NUMBER: W81XWH-14-1-0428

TITLE: Exploiting Tumor-Activated Testes Proteins To Enhance Efficacy of First-Line Chemotherapeutics in NSCLC

PRINCIPAL INVESTIGATOR: Angelique Whitehurst, PhD

CONTRACTING ORGANIZATION: University of Texas Southwestern Medical Center
                                                  Dallas, TX 75390

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                          Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE		*Form Approved* *OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE

Exploiting Tumor-Activated Testes Proteins To Enhance Efficacy of First-Line Chemotherapeutics in NSCLC

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0428

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Angelique Whitehurst, PhD

E-Mail:Angelique.Whitehurst@utsouthwestern.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas
  Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, TX 75390-9020

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Cancer Testis Antigens (CTAs) are a group of proteins whose expression is restricted to reproductive tissues (testis and ovaries), but frequently activated in Non Small Cell Lung Cancer (NSCLC). A number of these CTAs are essential for meiosis during spermatogenesis. Their deletion in mice leads to infertility due to an inability to repair DNA Double Strand breaks (DSB) during homologous recombination in meiosis. DSBs frequently occur in the tumorigenic environment due to environmental insults such as hypoxia and reactive oxygen species. We hypothesize that CTAs promote repair of these DSB in NSCLC and are essential for tumor cell survival. To evaluate this hypothesis, we are investigating the biochemical, cell biological and in vivo activity of CTAs in NSCLC. We have identified CTAs that are essential for DNA DB in NSCLC and which are also essential to survival of these cells in vitro. We find that the expression of meiotic CTAs appears to be a marker for the inactivation of metabolic pathways that lead to the generation of DNA-damage species. These findings are under testing in vivo. The physical interaction network of CTAs and identification of additional CTAs that may mediate sensitivity to DNA-damage inducing drugs is under investigation.

15. SUBJECT TERMS
Cancer Testis Antigen (CTA), Fanconia-Anemia (FA), DNA Damage, Genomic Instability, DNA Double Strand Break (DSB)

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	**b. ABSTRACT** Unclassified	**c. THIS PAGE** Unclassified	Unclassified	11	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

1. **INTRODUCTION:** This proposal is focused on elucidating the function of Cancer-Testes Antigens (CTAs) in Non-Small Cell Lung Cancer. CTAs are genes whose expression is restricted to the testes but frequently reactivated in many tumor types. The purpose of this proposal is to elucidate the contribution of CTAs to the maintenance of genomic integrity at the molecular level and to examine whether CTAs modulate sensitivity to chemotherapy. To do this, a cohort of meiotic CTAs will be examined for their functional contribution to the activation of DNA damage repair pathways. These CTAs, TEX15, HORMAD1 and SYCP1 were implicated as important to NSCLC in preliminary studies. In addition, physical interactions between CTAs and components of the DNA repair machinery will be assessed. In vitro and in vivo studies will be used to assess the contribution of CTAs to tumor cell viability and chemosensitivity. A larger scale analysis of whether additional CTAs contribute to chemotherapeutic sensitivity will also be carried out.

2. Keywords:
   o Cancer Testis Antigen (CTA)
   o Fanconia-Anemia (FA)
   o DNA Damage
   o Genomic Instability
   o DNA Double Strand Break (DSB)

3. Accomplishments
   - **What were the major goals and objectives of the project?**

   0-12 months:
   1) To analyze the FA pathway in CTA-depleted lung cancer cells (Vaziri Lab)
   2) Complete Lentiviral-mediated CTA overexpression in HBE cells and analyze the FA pathway. (Vaziri Lab)
   3) Complete dose curve and transfection optimization for a panel of NSCLC cell lines. (Whitehurst Lab)
   4) Perform sensitivity assays for TEX15, SYCP1 and HORMAD1. (Whitehurst Lab)
   5) Obtain IACUC approval for in vivo studies. (Whitehurst Lab)
   6) Develop shRNA stable cell lines. (Whitehurst Lab)
   7) Initiate in vivo studies. (Whitehurst Lab)

   12-24 months:
   8) Analyze ectopically-expressed epitope-tagged CTA in lung cancer cell lines. (Vaziri Lab)
   9) Immunopurification and proteomic analysis of CTA complexes in cancer cells. (Vaziri Lab)
   10) Screen for CTAs contributing to DNA damage. (Whitehurst Lab)
   11) Continue in vivo studies. (Whitehurst Lab)

   - **What was accomplished under these goals?** (Since this is a CO-PI grant, the descriptions are split into Aim 1 (Vaziri) and Aim 2 (Whitehurst) for the accomplishment section.

   AIM 1: For each major goal proposed in SA1, major activities, specific objectives, and significant results/key outcomes are as follows:

Task: Analysis of FA pathway in CTA-depleted lung cancer cells (1.1)
Major activities: To deplete CTAs from cultured lung cancer cells (using siRNA), then analyze the effects of CTA loss on the integrity of different phases of DNA damage signaling, recruitment of DSB repair factors to damaged chromatin, and selection of DNA DSB repair pathway.
Specific objectives: To identify the specific phase(s) of DSB signaling and the sub-pathway(s) of DSB repair influenced by CTA expression.

Significant Results: Our comprehensive analysis of signaling events in CTA-expressing and CTA-depleted cultures indicates that CTA status does not influence the initiation of checkpoint protein kinase- and E3 ubiquitin ligase-mediated signal transduction cascades that are normally incited by DSB. However, CTA-depleted cancer cells show lesions in the chain of canonical signaling events distal to DSB resection. In particular, chromatin-binding of the DNA repair proteins that mediate strand invasion and Displacement-loop (D-loop) formation during Homologous Recombination (HR) is reduced in CTA-depleted cells. Consistent with a potential role for CTA in DSB repair, HR reporter assays (in which repair of a targeted strand break reconstitutes an intact GFP allele) show that CTA-depletion leads to reduced HR activity and radiosensitivity (**Fig. 1**). In similar assays that measure DSB repair via Non-Homologous End Joining (NHEJ), CTA depletion did not affect DNA repair activity (**Fig 1**). Therefore, the significant result of these studies is that CTAs can promote DNA DSB repair and radioresistance via HR in lung cancer cells.
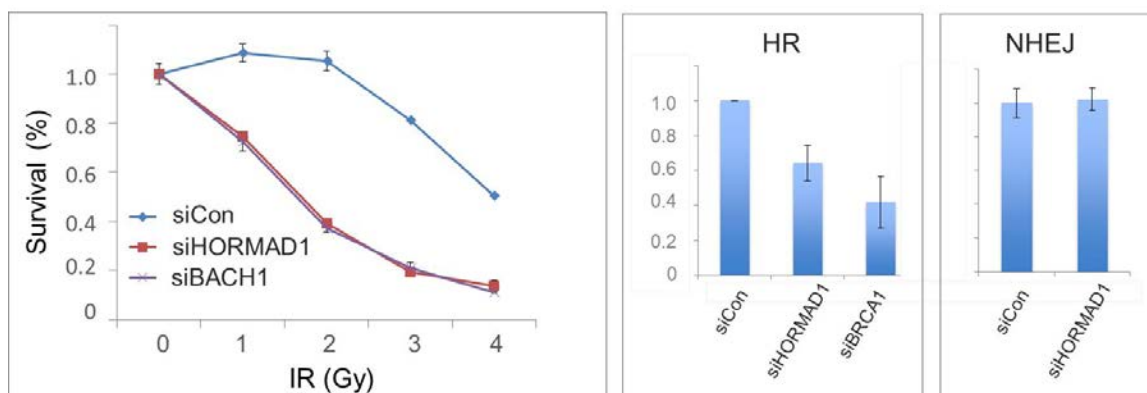

Figure. 1 Cells depleted of the CTA HORMAD1 are radiosensitive (**left panel**) and have reduced HR activity (but intact NHEJ) in DNA repair reporter assays (**right panels**).

Lentiviral CTA over-expression in HBE cells and analysis of FA pathway (1.1)
Major activities: To ectopically express CTA in non-transformed lung cells, then determine effects of CTA overexpression on the FA-mediated sub-pathway of HR.
Specific objectives: The objective of these experiments is to determine whether aberrant expression of individual CTAs in non-malignant cells is sufficient to activate DSB repair (thereby explaining the CTA-dependencies we have observed in siRNA experiments using cancer cells).
Significant results: Ectopic expression of CTAs in non-transformed cells did not affect any aspect of DSB signaling or DSB repair (as measured using reporter assays) that was tested. This result indicates that CTAs alone are insufficient to activate the core components of the DSB repair pathways. We infer that the CTA-dependency of DSB repair in lung cancer cell lines involves a broad program of events and alterations, acquired during tumor progression, that lead to CTA-addiction for DNA repair. Experiments in SA1.2 (which will identify the protein interaction network for CTA) seek to define the putative factors with which CTA cooperate to promote DSB repair.

Task: Analysis of ectopically-expressed epitope-tagged CTAs in lung cancer cells
Major activities: To express epitope-tagged fluorescent CTA in lung cancer cells and determine the basis for dynamic regulation of CTA distribution in response to DSB.
Specific objectives:
The objective of these experiments is to elucidate the putative mechanisms by which CTAs are recruited to the vicinity of DNA breaks to facilitate processing and repair of damaged DNA.
Significant results:

The significant result of these experiments is that CTAs can redistribute to 'Ionizing Radiation Induced Foci' (IRIF, large nuclear structures presumed to represent sites of DNA damage processing) and co-localize with known components of the DSB signaling pathway (such as the modified histone variant γH2AX) in response to irradiation (Fig. 2). These findings are fully consistent with results of 1.1, demonstrating that CTAs can respond dynamically to DNA damage by redistributing to DSB and contributing to DNA repair by HR.
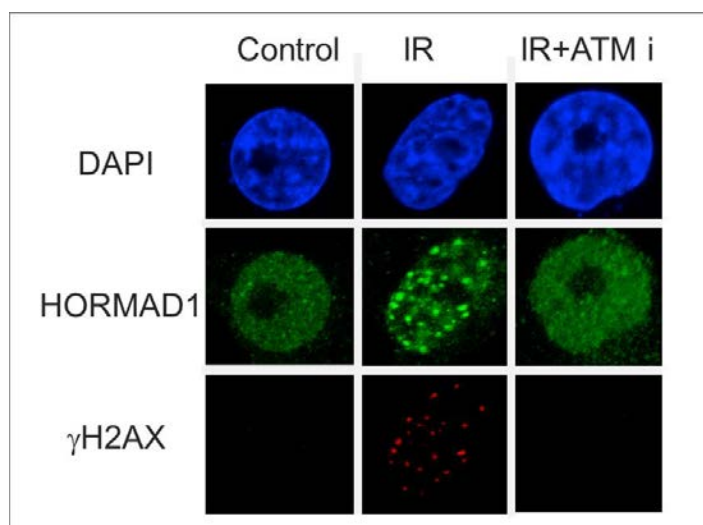

Figure. 2 The CTA HORMAD1 is pan-nuclear in undamaged cells **(left)** but redistributes and co-localizes with γH2AX foci following irradiation (middle panels). Pharmacological inhibition of the apical checkpoint kinase, ATM, abrogates the focal pattern of HORMAD1 and γH2AX distribution (right panels).

Task: Immuno-purification and proteomic analysis of CTA complexes in lung cancer cells
Major activities: To express epitope- tagged CTAs in cancer cells, immunopurify CTA complexes, and analyze by shotgun mass spectrometry.
Specific objectives: To identify putative CTA-associated proteins that mediate interactions with the DSB repair machinery.
Significant results: We have generated mammalian vectors for expression of HA-tagged CTA, used these to generate lung cancer cells lines that overexpress HA-CTA proteins modestly (~2-fold) above the endogenous proteins, and have optimized immunoprecipitation conditions that allow recovery and isolation of the ectopically-expressed CTA from chromatin fractions. In the next reporting period, these tools will be used to generate samples for proteomics studies.

AIM 2: The Whitehurst lab activities have been focused on Aim 2, in which we will assess dependency for survival of NSCLC on CTAs for survival or chemosensitivity of NSCLC in vitro and in vivo.
For each major goal proposed in SA2, major activities, specific objectives, and significant results/key outcomes are as follows:

Task: Complete dose curve and transfection optimization for a panel of NSCLC cell lines.
Major activities: To develop transfection protocols and drug sensitivity data for NSCLC expressing the CTA's, HORMAD1, TEX15 and SYCP1.

Specific Objectives. To develop a set of NSCLC cell lines for which high efficiency reverse transfection is possible and identify doses of sensitivity to allow for an analysis of requirements for HORMAD1, TEX15 and SYCP1 on tumor cell viability.

Significant results: We performed expression profiling on a panel of NSCLC cell lines and identified 6 (H1299, H661, H650, H460, H358 and HCC4017) cell lines for which we established high efficiency transfection conditions.

Task: Perform sensitivity assays for TEX15, SYCP1 and HORMAD1.
Major Activities: Using these cell lines from above, we used siRNA to deplete the expression of each of these CTAs and studied the consequences on tumor cell viability by measuring total ATP.
Specific Objective: To determine whether TEX15, SYCP1 or HORMAD1 are required for the viability of sensitivity of NSCLC to chemotherapeutic agents.
Significant Results: As seen in figure 3, inhibition of HORMAD1 led to a reduction in viability in nearly all cell lines tested. Depletion of TEX15 and SYCP1 were less penetrant that HORMAD1. Based on this finding and those mechanistic studies in the Vaziri lab (Figure 1&2), we prioritized HORMAD1 for further analysis. We did not observe a significant sensitization to a DNA damage agent for HORMAD1, camptothecin. Furthermore, in a panel of over 50 NSCLC cells for which HORMAD1 expression was characterized, we did not observe any correlation of expression with sensitivity to the DNA damage agent Cisplatin (p=0.65, see Figure 3). As we considered this possible outcome, we next leveraged a large cell line panel and drug sensitivity data set and found a striking correlation of HORMAD1 expression with resistance to Piericidin, an electron transport chain inhibitor. This finding suggests that HORMAD1 high expressing cells may not be able to tolerate high levels of electron transport chain activity as it would lead to the generation of reactive oxygen species that could damage DNA. Based on these findings, we have initiated in vivo studies on HORMAD1.
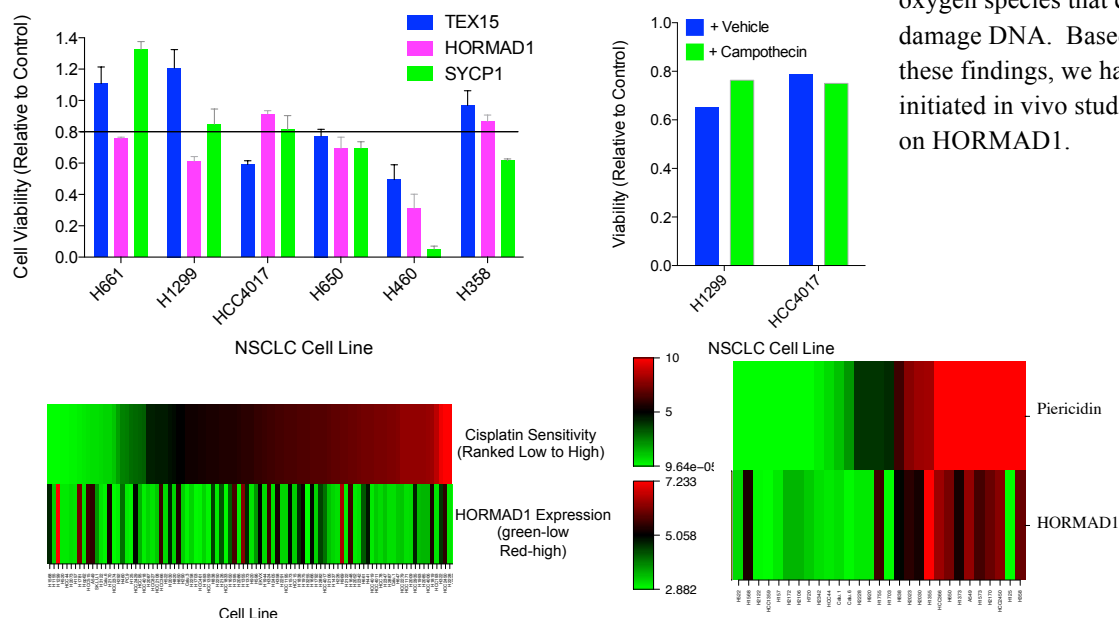

Figure 3: Top left: NSCLC were depleted of indicated CTAs and cell viability was measured. Line indicates the cutoff for sensitivity. Top Right: Camopthecin sensitivity assay for NSCLC cell lines. Bottom left: HORMAD1 expression and sensitivity of > 50 NSCLC cell lines to Cisplatin (ranked by Cisplatin sensitivity). Bottom Right: NSCLC cell lines (bottom) with HORMAD1 expression levels ranked for Piericidin sensitivity. Green is low expression/dose and Red is high expression/dose for bottom panels.

Task: Obtain IACUC approval for in vivo studies.

Major Activity – Create a protocol for approval by UTSW IACUC committee.

Specific Objective: To obtain approval for mouse studies to analyze the consequenes of CTA expression on tumor growth and chemosensitivity in vivo.

Significant Results: IACUC approval was obtained from UTSW for this study.

Task: Develop shRNA stable cell lines. (Whitehurst Lab)

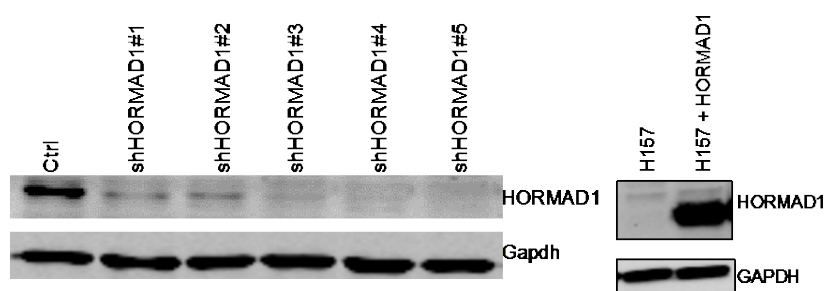**Major Activities:** To deplete cells of CTAs using shRNA.

Figure 4: Immunoblot of NSCLC cell lystaes with indicated shRNAs (left) or expression constructs (right).

Specific Objective: To attain cell lines where CTAs are stably depleted of overexpressed for in vivo and additional in vitro studies.

Significant Results: We used a well-established delivery method for the delivery of shRNA to NSCLC. We used 5 independent shRNA sequences to identify shRNAs that provide the largest depletion of CTAs. As demonstrated in Figure 4, we developed NSCLC cell lines in which CTAs are stably depleted. In addition, we established a NSCLC that is CTA negative and stably overexpressed HORMAD1. These cell lines will be used for subsequent in vitro and in vivo analysis

Task: Initiate in vivo studies. (Whitehurst Lab)

Major Activities: To monitor growth of NSCLC in mice following alteration of CTA protein expression.

Specific Objective: To determine whether CTAs can influence the growth of tumors in vivo.

Significant Results: Using the cell lines derived in Figure 4, we have begun tumor growth assays. In the next reporting period tumor growth and chemosensitivity will be monitored for these cells.

Task: Screen for CTAs contributing to DNA damage. (Whitehurst Lab)

Major Activities: To screen a large library of CTAs for their impact on DNA Damage.

Specific Objectives: To identify additional CTAs that may be contributing to the DNA damage response in NSCLC.

Significant Results: In the first phase of this analysis, we have begun developing a platform for screening analysis. We have identified cell lines as one for potential screening due to their ease of growth, effective plating, high transfection efficiency and frequent activation of CTAs. We are currently completing the assay development portion of our analysis and will screen the library of CTAs during the next reporting period.

Changes in Approach or Methods: As evident from the description above, there has been no significant change in approach or methods from the agency approved application or plan.

Adherence to original timetable: As expected, we have largely completed the analyses proposed in 1.1 and 2.1 within the first 12 months. We are slightly ahead of schedule in that we have already performed some of the subcellular distribution experiments (1.2) and generated tools and optimized experimental conditions necessary for proteomic studies (1.2). We have initiated the experiments proposed in 2.2 and 2.3, which will be conducted through the remainder of the granting period.

What opportunities for training and professional development has the project provided?

A post-doctoral fellow at UNC (Yanzhe Gao) performed the studies in support of SA1. His training activities included one-on-one work with Dr. Vaziri and acquisition of new imaging skills in deconvolution fluorescence microscopy (1.2). At UTSW, Jennifer Macion has been performing studies in support of Aim 2. This allowed her to gain new skills with respect to cell biology and screening analysis.

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

As described above, the bulk of the proposed work in 1.1 and 2.1 has been performed and generated results consistent with our central hypothesis that CTA facilitate DNA repair in lung cancer cells. The experiments in 1.2, to be conducted in the next period, seek to elucidate the mechanisms by which CTA respond to damaged DNA and interface with HR proteins to promote DSB repair and radioresistance. The experiments in aims 2.2. and 2.3 will further validate CTAs as essential for growth of tumors in vivo and identify additional CTAs that may be critical for DNA DSB. Specific tasks are below:

Analysis of ectopically-expressed epitope-tagged CTA in lung cancer cells (1.2)

We have observed dynamic redistribution of CTAs to IRIF in irradiated cells. We seek to elucidate the basis for recruitment of CTAs to damaged chromatin. We will test two non-mutually-exclusive hypotheses: (1) apical signals in the DSB signaling cascade directly regulate CTA redistribution to sites of damage. (2) Damaged chromatin and DNA repair intermediates are recognized by CTAs. In preliminary experiments in support of hypothesis 1, inhibition of checkpoint kinase signaling attenuated the redistribution of CTA to IRIF (**Fig. 2**), and some of the CTAs (including HORMAD1) contain consensus sites for DSB-responsive checkpoint kinases. Therefore, we plan further experiments to determine how specific ablation of apical DNA damage signaling events (using pharmacological inhibitors and siRNA) impact CTA regulation. We will also generate a series of CTA mutants (including phospo-mimetic and phosphorylation-resistant mutants) to enable a structure-function analysis of CTAs in relation to DNA damage signaling.

Immuno-purification and proteomic analysis of CTA complexes in lung cancer cells (1.2)

As described above, we have generated reagents necessary for proteomics studies to define the protein interaction network of CTAs. These experiments are a high priority since identification of CTA-associated proteins could reveal the molecular basis for interactions of CTAs with the DNA repair machinery. We have experience with the proposed experimental approach and anticipate that the shotgun mass-spectrometry experiments will be completed relatively quickly (~1 month). Depending on the number of CTA-complex members we identify, subsequent experiments to validate the specificity of interaction with CTA, and testing their potential roles in HR will most likely be the major activities in the next reporting period.

In vivo xenograft experiments: (2.2)

As described above, we have generated the cell lines with altered expression of CTAs necessary for in vivo studies. These experiments will first determine whether CTAs can contribute to tumorigenic growth and if changes that occur in vitro also may occur in vivo. We have initiated these animal studies, which will be completed over the next 12 months.

<u>Synthetic lethal screen for CT-antigens that modulate DNA Damage response. (2.3)</u>

As described above, we have identified nearly completed the assay development platform for the CTA screen. This platform should be finalized in the next 1-2 months and screening initiated (screening will take 2 weeks to complete). Subsequent screens and hit identification will also be performed during the next reporting period as planned .

4. **Impact**
   Nothing to report.

5. **CHANGES/PROBLEMS**

   Nothing to report.

6. **PRODUCTS:**

   Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort).

Name:	Cyrus Vaziri
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Wrote SA1 of original proposal and supervised experiments in support of SA1
Funding Support:	NIH awards and partial support from this award

Name:	Yanzhe Gao
Project Role:	Post-doc
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	11
Contribution to Project:	Dr. Gao performed all the experiments described under 'work accomplished'
Funding Support:	This award and Start-up funds to Vaziri from UNC

Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Wrote SA1 of original proposal and supervised experiments in support of SA1
Funding Support:	This award, federal, private and institutional funds.

Name:	Jennifer Macion
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	8
Contribution to Project:	Jennifer Macion worked on the experiments in Aim 2.
Funding Support:	This award, federal and private foundation funds.

Has there been a change in the active other support of the PD/PIs since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

Partner's Contribution to the project: Collaboration

8. **SPECIAL REPORTING REQUIREMENTS:** None

9. **APPENDICES:** None (Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.)